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Streptomycin and its analogues are potent inhibitors of the hypotonicity-induced Ca^{2+} entry and Cl^- channel activity

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Abstract **Streptomycin is a common antibiotic used in culture media. It is also a known blocker of stretch-activated and mechanosensitive ion channels in neurons and cardiac myocytes. But very little information is available on its effect in the regulation of epithelial ion channels. Osmotic swelling is a kind of mechanical stretch. The opening of stretch-activated Ca^{2+} channels contributes to hypotonicity-induced Ca^{2+} influx which is necessary for the activation of volume-regulated Cl^- channels in human cervical cancer cells. This study aimed to investigate the role of streptomycin in cell volume regulation. Treatment of cervical cancer SiHa cells with streptomycin and its analogues (gentamicin and netilmicin) did not affect the basal cytosolic Ca^{2+} ($[Ca^{2+}]_i$) level. But it attenuated the hypotonicity-stimulated increase of $[Ca^{2+}]_i$ in a dose-dependent manner with half-maximal inhibitory concentrations (IC_{50}) of 25, 90 and 200 μM for streptomycin, gentamicin and netilmicin, respectively, when measured at room temperature. In contrast, under free extracellular Ca^{2+} condition, hypotonic stress only induced a small, progressive increase of $[Ca^{2+}]_i$, while 500 μM streptomycin did not affect this Ca^{2+} signaling. Streptomycin and its analogues (gentamicin and netilmicin) also inhibited the activation of volume-regulated Cl^- channels in a dose-dependent manner with IC_{50} of 30, 95 and 250 μM at room temperature, respectively. Chronic culture with 50 μM streptomycin downregulates the activity of volume-regulated Cl^- channels and retards the process of regulatory volume decrease in SiHa cells and MDCK cells. We suggest that using cells chronically cultured with streptomycin to study epithelial ion channels risks studying cellular and molecular pathology rather than physiology.**

Key words: Streptomycin; Volume regulation; Cervical cancer cell

1. Introduction

Homeostasis of cell volume is a fundamental cellular property. Cells defend themselves against hypotonic stress by losing solutes together with osmotically obligated water, a process termed regulatory volume decrease (RVD). The principal solutes lost in RVD are K^+, Cl^- and a variety of largely uncharged or zwitterionic organic solutes, such as taurine [1]. The predominant pathway for RVD in most cell types is the opening of separate K^+ and Cl^- channels [2,3]. The volume-regulated Cl^- channel has attracted wide interest because of its role in several important cellular functions, including volume regulation, control of membrane potential, pH homeostasis, transport of amino acids and cell proliferation [4,5]. We have demonstrated previously that the activity of volume-regulated Cl^- channels which leads to Cl^- and taurine efflux was strongly upregulated during human cervical carcinogenesis [6,7]. The cell cycle progression of cervical cancer cells was also accompanied by differential activities of volume-regulated Cl^- channels [8].

The importance of Ca^{2+} signalling in the RVD process has been demonstrated in many cell types. In cervical cancer cells, hypotonic cell swelling triggers an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that is deemed responsible for the subsequent RVD [9–11]. Osmotic swelling of cervical cancer cells induces a $[Ca^{2+}]_i$ transient, which is an initial steep rise of $[Ca^{2+}]_i$ and is followed by a decay to reach a plateau level. The initial peak rise of $[Ca^{2+}]_i$ likely arises from the activation of stretch-activated Ca^{2+} channels which is sensitive to Gd^{3+} [11]. The plateau level of $[Ca^{2+}]_i$ is mainly maintained by Ca^{2+} release from intracellular stores. This hypotonicity-induced Ca^{2+} signalling is necessary for the activation of volume-regulated K^+ channels, Cl^- channels and osmosensing signal pathways, indicating that this $[Ca^{2+}]_i$ transient is critical for a normal RVD response of human cervical cancer cells.

Streptomycin is a common antibiotic used in culture media. It has been demonstrated that streptomycin and its analogues can effectively inhibit the activation of several types of ion channels, such as P/Q-type Ca^{2+} channels in mammalian neurons [12], maxi-K^+ channels in cochlear efferent nerve terminals [13], voltage-dependent Ca^{2+} channels in vertebrate nerve terminals [14], and stretch-activated ion channels in hair cells [15] and ventricular myocytes [16]. However, very little information is available on its effect in the regulation of epithelial ion channels. Osmotic swelling is a kind of mechanical stretch and the activation of stretch-activated Ca^{2+} channels is proposed to be responsible for hypotonicity-induced Ca^{2+} influx [17,18]. It is likely that streptomycin, which is a known blocker of stretch-activated and mechanosensitive ion channels in neurons and cardiac myocytes, can decrease the hypotonicity-induced Ca^{2+} entry, thus inhibiting the osmosensing signal pathways and the activation of volume-regulated Cl^- chan-

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Abbreviations: RVD, regulatory volume decrease; $[Ca^{2+}]_i$, cytosolic Ca^{2+}

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nels. Therefore, the specific aim of the present study was to determine whether streptomycin can affect Ca^{2+} signalling and volume-regulated Cl^- channels in swollen cervical cancer cells. The results demonstrate that streptomycin and its analogues are potent inhibitors of the hypotonicity-induced Ca^{2+} entry and Cl^- channel activity. More importantly, the RVD process is retarded when cells are chronically cultured with streptomycin.

2. Materials and methods

2.1. Cell culture

The human cervical cancer cell line (SiHa) and Madin–Darby canine kidney (MDCK) cell line were obtained from the American Type Culture Collection (Manassas, VA, USA). SiHa and MDCK cells were maintained at 37°C in a CO_2–air (5%–95%) atmosphere and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen). In some experimental conditions, 80 μg/ml streptomycin (Sigma, St. Louis, MO, USA) was added to the culture medium.

2.2. Chemicals and solutions

All chemicals were obtained from Sigma. The isotonic medium (300 ± 3 mosm/l) contained (in mM): NaCl 100, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.5, glucose 10, HEPES 10 and mannitol 70, titrated to pH 7.4 with NaOH. The components of the hypotonic medium were the same as those of the isotonic medium except that mannitol was omitted, resulting in a 23% hypotonicity (230 ± 3 mosm/l). In the experiments of altering extracellular Ca^{2+}, 1.5 mM EGTA was added to the bath solution and 1.5 mM $CaCl_2$ was omitted. To measure the activity of volume-regulated Cl^- channels, KCl was replaced by CsCl in the media and the pipette solutions contained (in mM): CsCl 40, Cs-aspartate 100, $MgCl_2$ 1, $CaCl_2$ 1.93, EGTA 5, ATP 2, GTP 0.5, HEPES 5. The pipette solutions were adjusted to pH 7.2 with CsOH.

2.3. $[Ca^{2+}]_i$ measurement with fura-2

$[Ca^{2+}]_i$ was measured at room temperature with the fura-2 fluorescence ratio method on a single cell fluorimeter as previously described [19]. In brief, cells attached on a coverslip were loaded with 2 μM fura-2/acetoxymethyl ester (fura-2/AM) in DMEM culture medium at room temperature for 40 min and then at 37°C for 20 min. After loading, cells were washed three times with phosphate-buffered saline. After washing, coverslips were placed on the stage of an Olympus IX71 inverted microscope equipped with a xenon illumination system and an IMAGO CCD camera (Till Photonics, Grafelfing, Germany). The excitation wavelength was alternated between 340 nm (I_{340}) and 380 nm (I_{380}) using the Polychrome IV monochromator (Till Photonics). The fluorescence intensity was monitored at 510 nm, stored digitally and analyzed by the program of TILLvisION 4.0 (Till Photonics). $[Ca^{2+}]_i$ was calculated from I_{340}/I_{380} ratio using the equation [20]:

$$[Ca^{2+}]_i = K_d \times (F_{min}/F_{max}) \times [(R - R_{min})/(R_{max} - R)]$$

where K_d is the dissociation constant for fura-2 in the cytosol (250 nM), F_{min} and R_{min} are the 380 nm fluorescence intensity and I_{340}/I_{380} ratio at low $[Ca^{2+}]_i$, respectively. F_{max} and R_{max} are the 340 nm fluorescence intensity and I_{340}/I_{380} ratio at high $[Ca^{2+}]_i$, and R is the I_{340}/I_{380} ratio recorded during experiments. Calibration measurements of F_{min} and R_{min} were performed after incubating cells for 10 min in nominally Ca^{2+}-free isotonic solution containing 3 mM EGTA. Cells were then superfused with isotonic solution containing 1 μM thapsigargin, 5 μM ionomycin and 10 mM Ca^{2+} to evaluate F_{max} and R_{max}.

2.4. Electrophysiological measurements

The whole-cell mode of the patch-clamp technique was used to measure membrane currents at room temperature (22–25°C) or at 37°C as previously described [8,11]. The current–voltage relationship and time course of volume-regulated Cl^- current were obtained from either a ramp or a step protocol. The ramp protocol consisted of: a step to −80 mV for 0.4 s and followed by a 1.3 s linear voltage ramp to +80 mV, after which the potential was stepped back to the holding potential of −20 mV. This voltage protocol was repeated every 15 s from a holding potential of −20 mV. The step protocol consisted of a 1 s voltage step, applied every 15 s from a holding potential of −20 mV to test potentials from −80 to +80 mV with an increment of 20 mV. Currents were sampled at 1 ms intervals. Data from electrophysiological experiments were digitized and analyzed using pCLAMP software (Version 6.0.3, Axon Instruments, Union City, CA, USA).

2.5. Measurements of cell volume

Cell volume was measured at room temperature (22–25°C) as described previously [11,21]. Cells were viewed with magnification up to ×400 by an Olympus IX71 inverted microscope, which was equipped with Hoffman modulation optics (Olympus, Tokyo, Japan). In order to monitor the change of cell size, the microscope was coupled to a video camera system and the images were recorded in real time and stored on a video cassette recorder. Images were then analyzed by Image-Pro Express (Version 4.0, Media Cybernetics, MD, USA). The majority of cells observed were spheroid and the relative volume change (V/V_o) was calculated from the cross-sectional surface area at the beginning (S_o) of the experiment and during (S) the experiments from the relation: $V/V_o = (S/S_o)^{3/2}$ [11,21]. Data are presented as a percentage of the starting volume (V/V_o), as a function of time. The validity of this approach to measure cell volume has been demonstrated in mouse thymocytes [19], renal A6 cells [22], human cervical cancer cells [11] and MDCK cells [21].

2.6. Statistics

All values in the present study are reported as means ± S.E.M. Student's pair or unpaired t-test was used for statistical analyses. Differences between values were considered significant when $P < 0.05$. The dose–inhibition curves for the drug effects were fitted into the following equation:

$$\%\ \text{inhibition} = \frac{100}{1 + (IC_{50}/C)^P}$$

in which C is the drug concentration, P the Hill coefficient, and IC_{50} the drug concentration needed for half-maximal inhibition.

3. Results

3.1. Streptomycin attenuates the hypotonicity-induced Ca^{2+} signaling

The Ca^{2+} signaling in response to hypotonicity was investigated in cervical cancer SiHa cells loaded with the Ca^{2+}-sensitive dye fura-2/AM. Superfusion of SiHa cells with the hypotonic solution elicited a rapid rise of $[Ca^{2+}]_i$ from the basal level of 100 ± 8 nM to a peak of 550 ± 10 nM ($n = 50$). The initial steep rise of $[Ca^{2+}]_i$ was followed by a decay to a plateau of 120 ± 5 nM (Fig. 1A). Treatment of SiHa cells with streptomycin did not affect the basal $[Ca^{2+}]_i$ level. But it attenuated the hypotonicity-stimulated increase of $[Ca^{2+}]_i$ in a dose-dependent manner (Fig. 1A). For example, streptomycin at 50 and 500 μM inhibited the peak rise of $[Ca^{2+}]_i$ from 550 ± 10 nM to 250 ± 8 nM ($P < 0.01$, $n = 50$) and 130 ± 5 nM ($P < 0.001$, $n = 50$), respectively.

Subsequent experiments were done by altering extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). Under the condition of $[Ca^{2+}]_o$ near 0 mM, hypotonic stress only induced a small, progressive increase of $[Ca^{2+}]_i$, from a basal level of 50 ± 3 to a plateau of 110 ± 6 nM ($P < 0.01$, $n = 30$), while 500 μM streptomycin did not affect this Ca^{2+} signaling. These findings indicate that streptomycin inhibited Ca^{2+} entry from the extracellular medium, but did not affect the mobilization of Ca^{2+} from intracellular stores. Gentamicin and netilmicin, the analogues of streptomycin, also show the dose-dependently inhibitory effects on hypotonicity-induced Ca^{2+} signalling. The IC_{50} to inhibit the initial peak rise of $[Ca^{2+}]_i$ was 25, 90 and 250 μM for streptomycin, gentamicin and netilmicin, respectively (Fig. 1B).

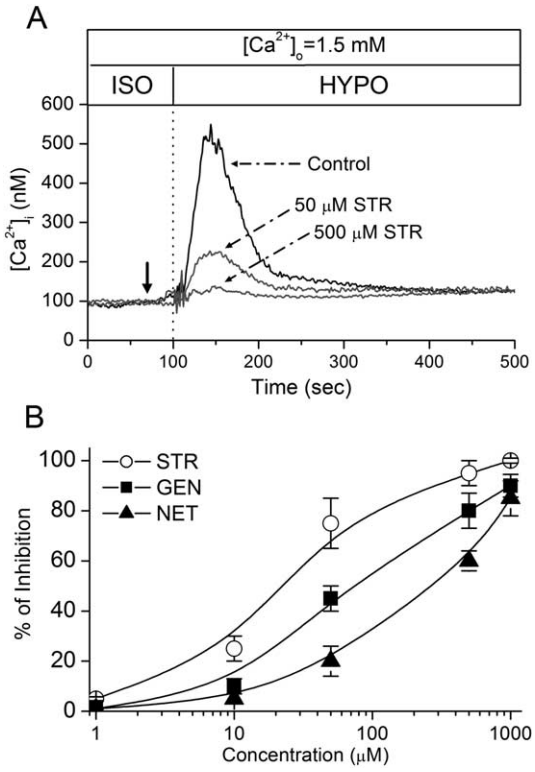

Fig. 1. Streptomycin and its analogues attenuate Ca^{2+} signalling in swollen cervical cancer SiHa cells. A: A representative recording of 50 similar traces at room temperature showing the changes of $[Ca^{2+}]_i$ evoked by a hypotonic solution containing 1.5 mM Ca^{2+} ($[Ca^{2+}]_o$) in the presence or absence of streptomycin. B: Dose–response curves for the inhibition of the initial peak rise of hypotonicity-induced $[Ca^{2+}]_i$ by streptomycin, gentamicin and netilmicin. Each point represents the mean ± S.E.M. ($n = 50$). Solid arrows indicate the time point when streptomycin was added. ISO: 300 mosm/l; HYPO: 230 mosm/l. STR: streptomycin; GEN: gentamicin; NET: netilmicin.

3.2. The activation of volume-regulated Cl^- channels is sensitive to streptomycin

We have previously demonstrated that hypotonicity-induced Ca^{2+} entry is necessary for the activation of volume-regulated Cl^- channels which play a leading role in the RVD of cervical cancer cells [9,11]. As shown in Fig. 1, streptomycin could significantly attenuate the hypotonicity-induced Ca^{2+} signalling. Thus it is likely that streptomycin can affect the activation of volume-regulated Cl^- channels. Whole-cell voltage-clamp recordings were therefore obtained from SiHa cervical cancer cells to study the effect of streptomycin on the activity of volume-regulated Cl^- channels. Membrane currents recorded during the ramp protocol applied to SiHa cells in the isotonic solution were small (Fig. 2A, trace 1). Application of a hypotonic solution induced cell swelling which was accompanied by an activation of large outwardly rectifying currents (Fig. 2A, trace 2). The hypotonicity-induced current reversed close to the theoretical equilibrium potential for Cl^- ($E_{Cl} = -25$ mV), indicating that the volume-regulated currents were carried mainly by Cl^- (Fig. 2A). As depicted in Fig. 2A (trace 3) and 2B, 50 μM streptomycin induced a fast, potent and reversible inhibition of volume-regulated Cl^- channels. In addition, streptomycin and its analogues (gentamicin and netilmicin) inhibited the amplitude of volume-regulated Cl^- channels in a dose-dependent manner with IC_{50} of 30, 95 and 300 μM, respectively.

To further dissect the effect of streptomycin on the activation of volume-regulated Cl^- channels, membrane currents were recorded during a step protocol applied to SiHa cells. The membrane currents in isotonic solution were small and time-independent (Fig. 3A). Application of a hypotonic solution activated large outwardly rectifying currents which became time-dependently inactivated at membrane potential more positive than +80 mV. Intracellular application of 50 μM streptomycin via pipette solutions did not produce either a suppression of basal membrane current or a decrease in the amplitude of volume-regulated Cl^- channels (Fig. 3B). Only applying streptomycin extracellularly can effectively inhibit the activation of volume-regulated Cl^- channels (Fig. 3).

3.3. Downregulation of volume-regulated Cl^- channels and retarded RVD in cells cultured with streptomycin

Streptomycin is a common antibiotic used in the culture medium. The dosage usually ranges from 50 to 100 μg/ml (approximately 35–70 μM). Referring to Figs. 1C and 2C,

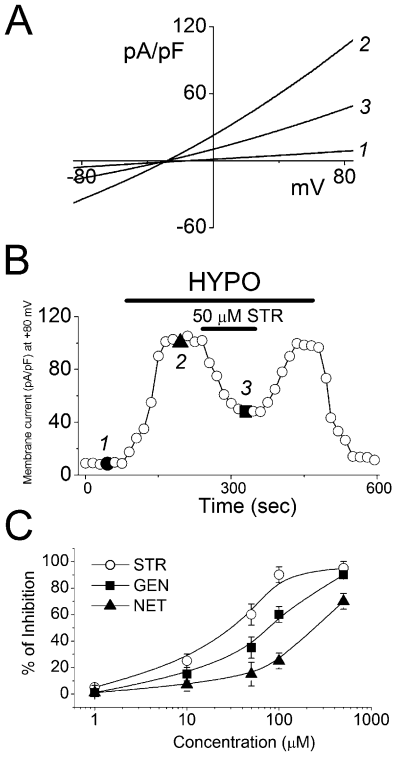

Fig. 2. Effect of streptomycin and its analogues on the volume-regulated Cl^- currents of human cervical cancer SiHa cells. A: Representative recordings of volume-regulated Cl^- currents from ramp protocol measured at room temperature. Trace 1: basal membrane current recorded in the isotonic solution; trace 2 and 3: currents recorded after perfusion with hypotonic solution in the absence or presence of 50 μM streptomycin, respectively. B: Time course of membrane currents activated at +80 mV. Data points were obtained from the voltage ramp protocol that was applied every 15 s. The labeled points correspond to the current traces recorded in A. Coarse horizontal bar: application of hypotonic solution (HYPO) or 50 μM streptomycin. C: Dose–response curves of streptomycin and its analogues on the inhibition of the volume-regulated Cl^- currents measured at +80 mV. Each point represents the mean ± S.E.M. ($n = 12$). HYPO: 230 mosm/l. STR: streptomycin; GEN: gentamicin; NET: netilmicin.

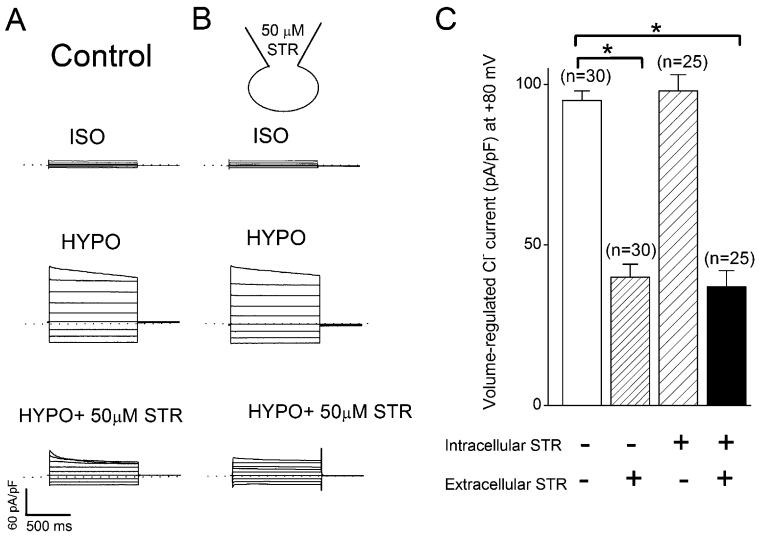

Fig. 3. Streptomycin inhibits the activation of the volume-regulated Cl^- current only in extracellular application. Representative current traces during the voltage step protocol (step protocol) were recorded without (A, control group) or with intracellular dialysis of 50 μM streptomycin (B) measured at room temperature. The dotted line is the zero current level. C: Summary of volume-regulated Cl^- currents measured at +80 mV in different experimental conditions. The number of cells examined is indicated in parentheses beside each bar. $^*P < 0.01$, unpaired *t*-test. ISO: 300 mosm/l; HYPO: 230 mosm/l. STR: 50 μM streptomycin.

acute treatment in this range of streptomycin can inhibit 50–70% of the hypotonicity-induced Ca^{2+} entry and Cl^- channel activity. We thus investigated whether the chronic treatment of streptomycin could alter the regulation of volume-regulated Cl^- channels. To test this hypothesis, we compared the activity of volume-regulated Cl^- channels in cells cultured in media with or without 50 μM streptomycin (Fig. 4). In the control group, SiHa cells had a small background current density in the isotonic medium, averaging -7.0 ± 1.5 pA/pF at -80 mV and $+10 \pm 1.4$ pA/pF at +80 mV ($n = 35$). For SiHa cells cultured in medium containing 50 μM streptomycin for 2 days, the background current density was -5.8 ± 1.4 pA/pF at -80 mV and $+7.8 \pm 1.6$ pA/pF at +80 mV ($n = 30$). There was no significant difference in background isotonic current density between these two groups of cells ($P > 0.05$, unpaired *t*-test). The normalized volume-regulated Cl^- current was used to compare the activity of volume-regulated Cl^- channels in various culture conditions. It was defined as the difference in current density between isotonic and hypotonic solutions and was expressed per unit membrane capacitance. For the control group, the normalized volume-regulated Cl^- current was 95 ± 3.8 pA/pF at +80 mV and -40 ± 2.9 pA/pF ($n = 35$) at -80 mV. Activity of volume-regulated Cl^- channels was still present in cells cultured with 50 μM streptomycin (Fig. 4A), but the current density was significantly decreased to 68 ± 2.1 pA/pF at +80 mV and -23 ± 1.0 pA/pF at -80 mV ($P < 0.01$, unpaired *t*-test, $n = 30$). In addition to altering the current amplitude, the activation rate of volume-regulated Cl^- channels was significantly decreased by streptomycin treatment (Fig. 4B). In control groups, exposure to hypotonicity induced an outwardly rectifying current with an activation rate of 1.06 ± 0.06 pA/pF/s ($n = 35$) at +80 mV. In contrast, SiHa cells treated with 50 μM streptomycin for 2 days expressed a slower current activation of 0.6 ± 0.07 pA/pF/s ($n = 30$, $P < 0.01$). These results indicate that the chronic treatment of streptomycin downregulates the activation of volume-regulated Cl^- channels. However, other fundamental characteristics of volume-regulated Cl^- channels were not affected, such as inactivation at potentials greater than +80 mV, anion selectivity $I^- > Br^- > Cl^-$ and sensitivity to the Cl^- channel inhibitors tamoxifen and NPPB.

We further studied whether chronic treatment with streptomycin altered the RVD process. As shown in Fig. 5, the typical volume response of SiHa cells to hypotonic stress can be divided into three phases: (1) an initial and rapid osmotic swelling, reaching a peak cell volume $19 \pm 3\%$ ($n = 60$) greater than the original cell size at about 2 min; (2) a rapid shrinkage in the following 3 min; (3) a more gradual decrease of cell volume to finally reach a plateau close to the original cell volume at about 7 min. In contrast to the typical RVD process, culture with 50 μM streptomycin for 2 days increased initial osmotic swelling, attenuated the shrinkage phase, and inhibited the gradual decrease in cell volume. This indicates that chronic exposure to streptomycin retarded the RVD process of cervical cancer cells.

The RVD process of SiHa cells is temperature-dependent. For example, the typical volume response of SiHa cells to hypotonic stress finishes within 10 min at room temperature and within 7 min at 37°C. The magnitude of the Ca^{2+} transient and Cl^- currents activated by hypotonic challenge is also markedly temperature-dependent, possibly reflecting the temperature dependence of enzymes involved in the intracellular signaling of cell volume regulation. The IC_{50} of streptomycin is certainly different under various temperature conditions. For example, streptomycin inhibits the activation of volume-regulated Cl^- channels with an IC_{50} of 30 μM at room temperature and 100 μM at 37°C.

We finally investigated the volume regulation of another type of epithelial cells (MDCK) which were chronically cultured with streptomycin. MDCK cells also need hypotonicity-induced Ca^{2+} entry for the normal RVD response and the activation of volume-regulated Cl^- channels [21]. As shown in Fig. 6A, treatment with 50 μM streptomycin for 2 days retarded the RVD process of MDCK cells. In patch-clamp

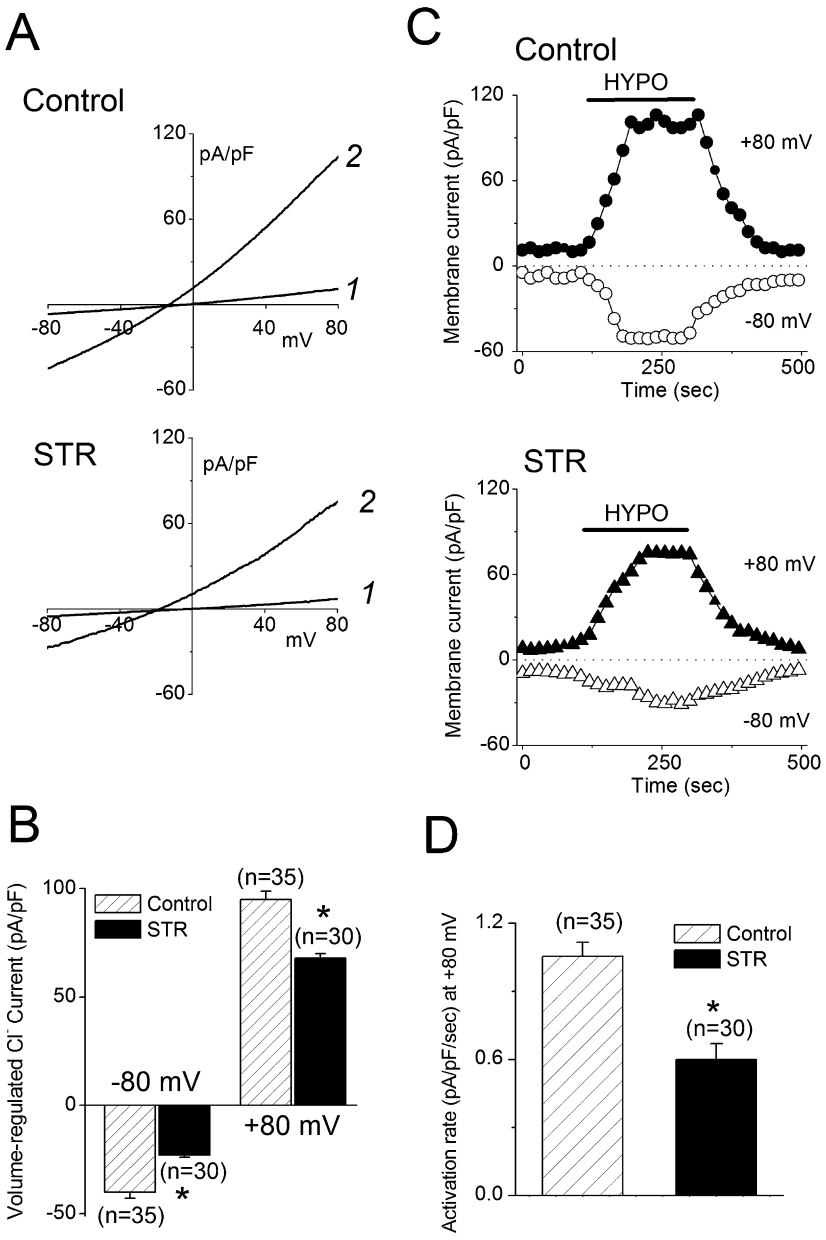

Fig. 4. Downregulation of volume-regulated Cl^- current in cells cultured with 50 μM streptomycin. A: Representative recordings of volume-regulated Cl^- currents measured at room temperature from a ramp protocol for cervical cancer SiHa cells in the normal culture condition (Control) or cultured in medium containing 50 μM streptomycin for 2 days. Trace 1: basal membrane current recorded in the isotonic solution; trace 2: currents recorded after perfusion with hypotonic solution. B: Normalized currents activated by hypotonicity measured at −80 mV or +80 mV. C: Time course of membrane currents activated at +80 mV or −80 mV. Data points were obtained from the voltage ramp protocol that was applied every 15 s. Coarse horizontal bar: application of hypotonic solution; horizontal line: zero current level. D: Summary of activation rate for volume-regulated Cl^- channels under various culture conditions. The number of cells examined is indicated in parentheses beside each bar. *$P < 0.01$, unpaired t-test. HYPO: 230 mosm/l. STR: cells cultured with 50 μM streptomycin for 2 days.

recordings, chronic exposure to 50 μM streptomycin significantly decreased the activation rate and amplitude of volume-regulated Cl^- channel in MDCK cells (Fig. 6B, $n = 15$).

4. Discussion

Streptomycin is a common antibiotic used in culture media. The dosage for cell culture ranges from 50 to 100 μg/ml (approximately 35–70 μM). The present study demonstrated that acute treatment with this concentration range of streptomycin can inhibit 50–70% of the hypotonicity-induced Ca^{2+} entry and Cl^- channel activity at room temperature. More importantly, chronic culture with 50 μM streptomycin downregulates the activity of volume-regulated Cl^- channels and retards the process of volume regulation in SiHa cells and MDCK cells. Alterations of cell volume and volume regulatory mechanisms participate in a wide variety of cellular functions including epithelial transport, metabolism, migration, cell proliferation, and cell death [4]. We suggest that using cells chronically cultured with streptomycin to study epithelial ion channels risks studying cellular and molecular pathology rather than physiology.

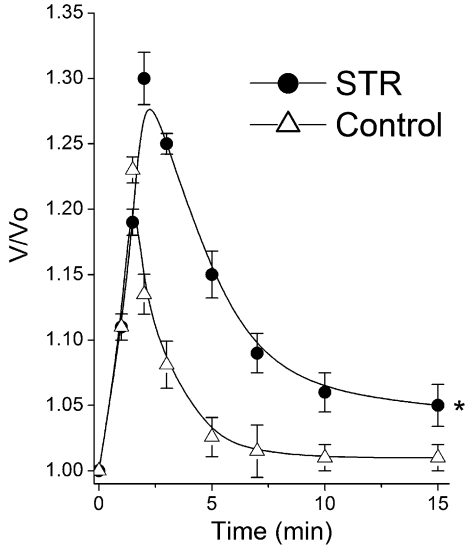

Fig. 5. Time courses of volume changes in cervical cancer SiHa cells following superfusion with hypotonic bath solution (230 mosm/l) of room temperature. The control group (Control) indicates cells in the normal culture condition for 2 days and STR indicates cells cultured with 50 μM streptomycin for 2 days. The *y*-axis (V/V_o) depicts cell volume at the indicated times divided by cell volume at zero time. Each point represents the mean ± S.E.M. ($n = 50$ cells). *$P < 0.01$, compared with the control group at 15 min.

Here we show that streptomycin inhibited Ca^{2+} entry from the extracellular medium, but did not affect the mobilization of Ca^{2+} from intracellular stores. In swollen cervical cancer cells, applying streptomycin extracellularly can effectively inhibit the activation of volume-regulated Cl^- channels, whereas intracellular perfusion of streptomycin does not affect this channel activity. Stretch-activated Ca^{2+} contributes to the hypotonicity-induced entry which is critically involved in the activation of volume-regulated Cl^- channels [9,11]. Therefore, the possible mechanism underlying the inhibitory effect of streptomycin on volume regulation is as follows. Streptomycin decreases hypotonicity-induced Ca^{2+} entry, thus inhibiting the osmosensing signal pathways and the activation of volume-regulated Cl^- channels. The process of RVD is therefore affected.

Ca^{2+} signaling plays a central role in activating and controlling RVD mechanisms in most cell types [4,23]. Osmotic swelling may increase $[Ca^{2+}]_i$ by both activation of Ca^{2+}-permeable channels in the cell membrane and Ca^{2+} release from intracellular stores. What is the mode of Ca^{2+} entry in response to hypotonic shock? The Ca^{2+}-permeable channel involved has not been characterized at the molecular level. The stretch-activated cation channels are presumably the routes for Ca^{2+} entry in hypotonic condition for most cell types [19,23]. P-type and L-type Ca^{2+} channels are suggested to be the channels responsible for hypotonicity-induced Ca^{2+} entry in a neuroblastoma cell line [24] and canine arterial myocytes [25], respectively. Recently, a new TRP (transient receptor potential)-like channel protein, OTRPC4, was proposed as a candidate for a molecular sensor that confers osmosensitivity on mammalian cells [26]. OTRPC4 is expressed at high levels in the kidney, liver and heart. It forms a Ca^{2+}-permeable, non-selective cation channel that exhibits spontaneous activity in isotonic media and is rapidly activated in hypotonic media. In HEK 293 cells expressing OTRPC4, changes in extracellular osmolarity of as little as 10% result in significant changes in the $[Ca^{2+}]_i$ level. However, this Ca^{2+} dependence is highly variable among cell types.

Cells can be grouped into three general categories based on the relative dependence of RVD on Ca^{2+} signalling: (1) cells that are highly dependent on extracellular Ca^{2+} and the activation of Ca^{2+} influx, supposedly reflecting activation of Ca^{2+} channels, such as observed in most cell types (reviewed in [23]); (2) cells that are not dependent on extracellular Ca^{2+} and Ca^{2+} influx but that require at least a certain basal $[Ca^{2+}]_i$ level or transient release of Ca^{2+} from internal stores, such as observed in the Ehrlich ascites tumor cells [27];

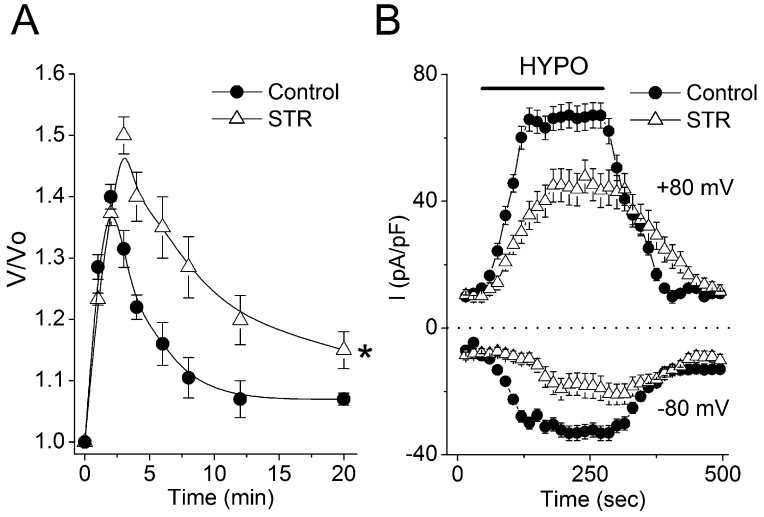

Fig. 6. Retarded RVD and downregulation of volume-regulated Cl^- channels for MDCK cells chronically treated with streptomycin. A: Time course of volume changes at room temperature in response to hypotonicity for MDCK cells cultured with or without 50 μM streptomycin (STR) for 2 days. The *y*-axis (V/V_o) depicts the cell volume at the indicated time divided by the cell volume at zero time. Each point represents the mean ± S.E.M. ($n = 50$ cells). *$P < 0.01$, comparing the volume ratio between groups at 20 min. B: Time courses of membrane currents activated at +80 mV or −80 mV for MDCK cells cultured with or without 50 μM streptomycin for 2 days. Data points were obtained from the voltage ramp protocol that was applied every 15 s. Horizontal bars indicate the application of hypotonic solution (HYPO; 230 mosm/l). Each point represents the mean ± S.E.M. ($n = 15$).

(3) cells that display little if any Ca^{2+} dependence, such as rat lymphocytes [28], and some species' red cells [4]. The site and mechanism of Ca^{2+} dependence of RVD are poorly understood. Some initial studies pointed to a possible direct control of K^+ and/or Cl^- channels by Ca^{2+} signalling to modulate KCl efflux and, hence, RVD. This viewpoint appears to be too simplistic [23]. As shown in our previous studies, the activation of volume-regulated Cl^- channels depends on a swelling-activated $[Ca^{2+}]_i$ transient in cervical cancer cells [9,11] and MDCK cells [21]. For example, in these two types of cells more than 85% of the activity of the volume-regulated Cl^- channel was suppressed when intracellular Ca^{2+} was buffered to near zero in the absence of extracellular Ca^{2+}, suggesting that hypotonicity-induced Ca^{2+} signaling is important for the activation of the volume-regulated Cl^- channel. However, simply increasing $[Ca^{2+}]_i$ in isotonic condition cannot activate this type of channel [29]. In swollen cervical cancer cells, several signal pathways were activated, such as the protein kinase Cα (PKCα) and extracellular signal-regulated kinase (ERK) 1/ERK2 mitogen-activated protein (MAP) kinase pathways. The activation of PKCα and ERK/ERK2 MAP kinase pathways requires Ca^{2+} entry and is critical for the activity of volume-regulated Cl^- channel [9,11]. This indicates the ion channels involved in RVD may not be directly Ca^{2+}-dependent and that some other regulatory process controlling the channel activation, perhaps a phosphorylation step, may be the Ca^{2+}-dependent events.

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References

[1] Kirk, K. (1997) J. Membr. Biol. 158, 1–16.
[2] Hoffmann, E.K. and Dunham, P.B. (1995) Int. Rev. Cytol. 161, 173–262.
[3] Okada, Y. (1997) Am. J. Physiol. 273, C755–C789.
[4] Lang, F., Busch, G.L., Ritter, M., Volkl, H., Waldegger, S., Gulbins, E. and Haussinger, D. (1998) Physiol. Rev. 78, 247–306.
[5] Nilius, B., Eggermont, J., Voets, T., Buyse, G., Manolopoulos, V. and Droogmans, G. (1997) Prog. Biophys. Mol. Biol. 68, 69–119.
[6] Chou, C.Y., Shen, M.R. and Wu, S.N. (1995) Cancer Res. 55, 6077–6083.
[7] Shen, M.R., Chou, C.Y. and Ellory, J.C. (2001) Pflügers Arch. 441, 787–795.
[8] Shen, M.R., Droogmans, G., Eggermont, J., Voets, T., Ellory, J.C. and Nilius, B. (2000) J. Physiol. 529, 385–394.
[9] Chou, C.Y., Shen, M.R., Hsu, K.S., Huang, H.Y. and Lin, H.C. (1998) J. Physiol. 512, 435–448.
[10] Shen, M.R., Chou, C.Y., Wu, M.L. and Huang, K.E. (1998) Cell Signal. 10, 113–120.
[11] Shen, M.R., Chou, C.Y., Browning, J.A., Wilkins, R.J. and Ellory, J.C. (2001) J. Physiol. 537, 347–362.
[12] Pichler, M., Wang, Z., Grabner-Weiss, C., Reimer, D., Hering, S., Grabner, M., Glossmann, H. and Striessnig, J. (1996) Biochemistry 35, 14659–14664.
[13] Takeuchi, S. and Wangemann, P. (1993) Hear. Res. 67, 13–19.
[14] Parsons, T.D., Obaid, A.L. and Salzberg, B.M. (1992) J. Gen. Physiol. 99, 491–504.
[15] Ricci, A.J., Crawford, A.C. and Fettiplace, R. (2000) J. Neurosci. 20, 7131–7142.
[16] Gannier, F., White, E., Lacampagne, A., Garnier, D. and Le Guennec, J.Y. (1994) Cardiovasc. Res. 28, 1193–1198.
[17] Chen, Y., Simasko, S.M., Niggel, J., Sigurdson, W.J. and Sachs, F. (1996) Am. J. Physiol. 270, C1790–C1798.
[18] Nakao, M., Ono, K., Fujisawa, S. and Iijima, T. (1999) Am. J. Physiol. 276, C238–C249.
[19] Ross, P.E. and Cahalan, M.D. (1995) J. Gen. Physiol. 106, 415–444.
[20] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[21] Shen, M.R., Yang, T.P. and Tang, M.J. (2002) J. Biol. Chem. 277, 15592–15599.
[22] Urbach, V., Leguen, I., O'Kelly, I. and Harvey, B.J. (1999) J. Membr. Biol. 168, 29–37.
[23] McCarty, N.A. and O'Neil, R.G. (1992) Physiol. Rev. 72, 1037–1061.
[24] Basavappa, S., Chartouni, V., Kirk, K., Prpic, V., Ellory, J.C. and Mangel, A.W. (1995) J. Neurosci. 15, 3662–3666.
[25] Kimura, M., Obara, K., Sasase, T., Ishikawa, T., Tanabe, Y. and Nakayama, K. (2000) Br. J. Pharmacol. 130, 923–931.
[26] Strotmann, R., Harteneck, C., Nunnenmacher, K., Schultz, G. and Plant, T.D. (2000) Nat. Cell Biol. 2, 695–702.
[27] Hoffmann, E.K., Simonsen, L.O. and Lambert, I.H. (1984) J. Membr. Biol. 78, 211–222.
[28] Grinstein, S. and Smith, J.D. (1989) Am. J. Physiol. 257, C197–C206.
[29] Shen, M.R., Furla, P., Chou, C.Y. and Ellory, J.C. (2002) Pflügers Arch. 444, 276–285.